Rapid Generation of a novel DPP-4 inhibitor with long-acting property: SAR study and PK/PD evaluation

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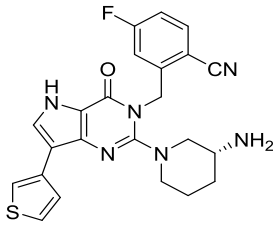
GRAPHIC ABSTRACT



124, with IC_{50} of 0.76 nM and competent *in vivo* efficacy with Trelagliptin 加体内药效图

Compound **124** with IC_{50} of 0.76 nM and competent *in vivo* efficacy with Trelagliptin.

ABSTRACT

Drug compliance is critical for the patients with chronic diseases like diabetes. In our continuous effort to find better glucose lowering agents, an exploration for long-acting DPP-4 inhibitor had been launched. Based on our previous reported compound **111** bearing a pyrrolopyrimidine scaffold, lead compound **114** (IC_{50} = 2.3 nM, $t_{1/2(rat)}$

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= 5.46 h) was rapidly determined with the pharmacokinetic superiority. Further SAR study indicated that the pyrrole ring was generally tolerable for variation, in which the β-substitution gave a better DPP-4 affinity. In depth evaluation on β position of pyrrole ring brought up with highly potent compound **124** (IC_{50} = 0.76 nM, $t_{1/2(rat)}$ = 7.89 h). *In vivo* pharmacodynamics tests demonstrated a similar or even slightly better sustained DPP-4 inhibition of compound **114** and **124** compared with the first marketed once-weekly drug Trelagliptin in this category, indicating that improvement of DPP-4 inhibitory activity or pharmacokinetic profile might be both feasible ways to rapid generation of long-acting DPP-4 inhibitors.

KEYWORDS

DPP-4 inhibitor; type 2 diabetes; SAR; long-acting; *in vivo* efficacy

1. Introduction

Diabetes mellitus is a chronic metabolic diseases characterized by hyperglycemia and has reached a world prevalence of 415 million patients. Moreover, this population is expected to rise rapidly to 642 million by 2040. Type 2 diabetes (T2D), which accounts for up to 90% of diabetes patients, is due to the insufficient response to insulin [1] [待完整后更换为 IDF Diabetes Atlas - 7th edition. Available from http://www.diabetesatlas.org/]. Undiagnosed T2D and the multisystem complications caused by hyperglycemia are the leading reason for patients' disability and mortality. Dipeptidyl peptidase-4 (DPP-4) inhibitor is a type of weight neutral and well tolerated glucose-lowering agents, functioned dominantly by inhibition of cleavage of glucagon like peptide-1 (GLP-1). GLP-1, an important incretin hormone, has multiple glucose regulation functions: stimulate insulin release in a glucose dependent way, increase the sensitivity of insulin, and reduce glucagon secretion [2, 3]. Currently, DPP-4 inhibitor are recommended as an add-on therapy with metformin, or as the first-line therapy in patients contradicted of metformin [4].

However, despite of many drugs available, poor adherence has led to the unsatisfying glycaemic control in around half of the T2D patients [5]. Combination treatment and reduction of dosing frequency are the common strategies to improve patients' adherence [6]. Thus long-acting glucose-lowering agents have been marketed successively, including once-weekly DPP-4 inhibitor. Trelagliptin and Omarigliptin were the first two long-acting DPP-4 inhibitor that received marketing authorization in Japan in 2015 (Fig. 1) [7, 8]. Up to data clinical trials of these two drugs demonstrated their superior efficacy to placebo and were not associated with any severe adverse

events [9]. Though comparing with the regular DPP-4 inhibitors, once-weekly Trelagliptin and Omarigliptin didn't show significantly better efficacy in term of glyceamic control [10, 11], and Omarigliptin was reported to be linked with potential safety issues [12][待完整后更换为 Advera Health Analytics. Pipeline drug evidence review: marizev (omarigliptin) vs. Januvia (sitagliptin). Available from: http://info.adverahealth.com/marizev-evidence-review [last accessed 25 July 2017], both manufacturers abandoned their marketing plans in other countries with the consideration of high financial costs [9]. Nonetheless, long-acting DPP-4 inhibitors still have the necessity to be go on developing for the sake of the fast escalating T2D patients, providing more choice for therapeutic drug treatment regardless of concerns [13].

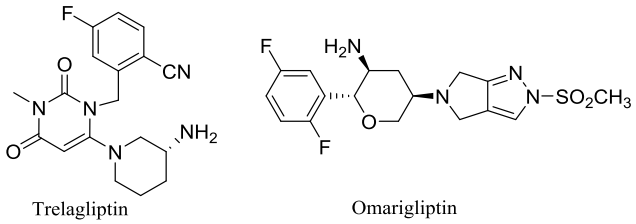


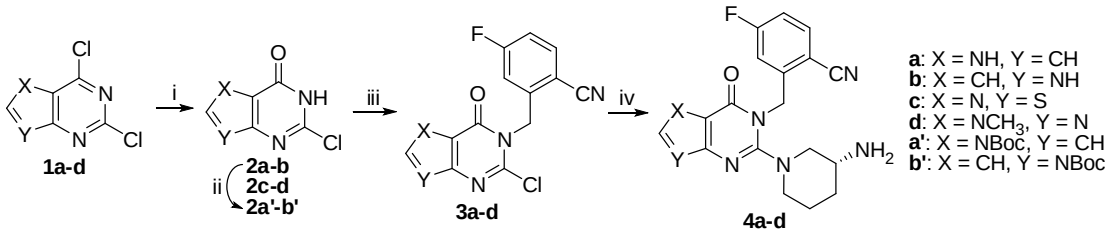
Figure 1. Marketed once- weekly DPP-IV inhibitors.

Previously, we reported a series of pyrrolopyrimine analogues based on a pharmacokinetic (PK) property-driven optimization. And the basal scaffold is represented by compound **111** (Fig. 2) [14]. In our continuous drug discovery effort for oral, potent, DPP-4 inhibitors with long-acting property, we started from the pyrrolopyrimine scaffold which bears a fine PK profile. Inspired by the discovery of Trelagliptin, 5-floro substitution was simply added to the cyanobenzyl group and generated the lead compound **114** (IC_{50}= 2.3 nM), which displayed a similar half-life with Trelagliptin in rat PK experiment. Expand structure-activity relationship (SAR) study was carried out around compound **114** and indicated that β-substitution on pyrrole ring brought improvement of DPP-4 affinity and was open to wild variation. Eventually the thienyl substituted compound **124** was proved to have a sustained in vivo DPP-4 inhibition in the pharmacodynamics (PD) assays, similar or slightly better than Trelagliptin.

2. Chemistry

The synthesis of compounds **4a-d** is outlined in Scheme 1. **1a-d** were hydrolyzed with aqueous sodium hydroxide to give **2a-d**, and **2a-b** were changed to **2a'-b'** by protection with Di-tert-butyl pyrocarbonate. Selective N-alkylation of **2a'-b'** and **2c-d** with 2-(bromomethyl)-4-fluorobenzonitrile provided precursor **3a-d,** The final

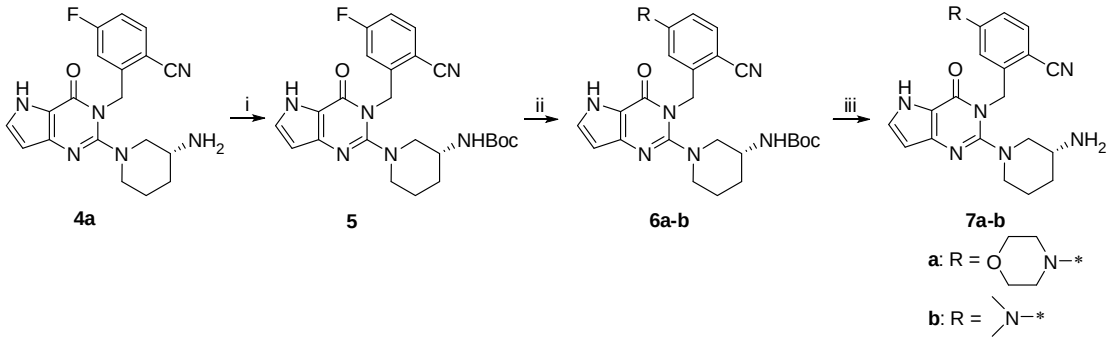
compounds **4a-d**, were obtained by the amination of the chloro-precursors **3a-d** with 3-(R)-aminopiperidine.



Scheme 1. Synthesis of compounds **4a-d**.

Reagents: (i) NaOH, H_2O, 100℃; (ii) Boc_2O, TEA, DMAP, DMF, rt; (iii) 2-(bromomethyl)-4-fluorobenzonitrile, NaH, LiBr, DMF/DME; (iv) 3-(R)-aminopiperidine, $NaHCO_3$, EtOH , 120℃.

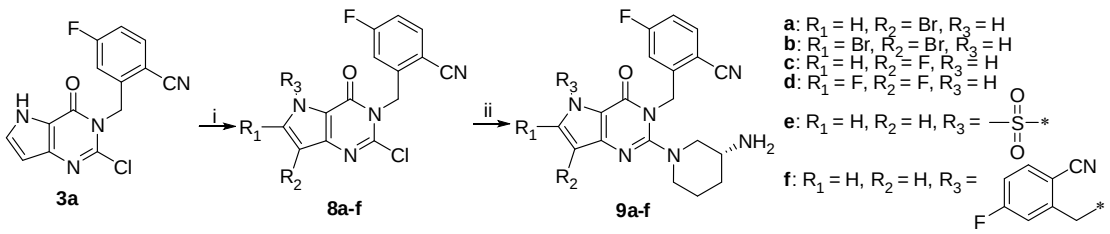
The synthesis of compounds **7a** and **7b** are outlined in Scheme 2. **6a** and **6b** were obtained by the amination with morpholino and dimethylamine respectively, of the fluoride **5** which were obtained by protection with Di-tert-butyl pyrocarbonate from compound **4a**. The final compounds **7a** and **7b**, were obtained by de-protection with HCl/MeOH solution from **6a** and **6b**.



Scheme 2. Synthesis of compounds **7a-b**.

Reagents: (i) Boc_2O, TEA, DCM, rt; (ii) Morpholino or dimethylamine, CuI, K_2CO_3, DMSO, 120℃; (iii) HCl/MeOH, rt.

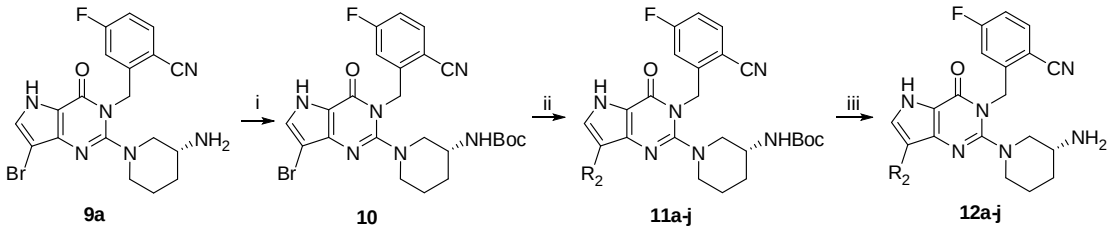
The synthesis of compounds **9a-f** are outlined in Scheme 3. Compound **3a** was changed to compounds **8a** and **8b** with N-bromosuccinimide (NBS) by bromination, and to compounds **8c** and **8d** with selectfluor by fluorination, and to compounds **8e** and **8f** with methanesulfonyl chloride and 2-(bromomethyl)-4-fluorobenzonitrile by Sulfonation and N-alkylation respectively. The final compounds **9a-f** were obtained by the amination of the chloro-precursors **8a-f** with 3-(R)-aminopiperidine.



Scheme 3. Synthesis of compounds **9a-f**.

Reagents: (i) **8a-b**: NBS, DMF, rt; **8c-d**: Selectfluor, $ZrCl_4$, CH_3CN, 80℃; **8e**: CH_3SO_2Cl, TEA, DMF, rt; **8f**: 2-(bromomethyl)-4-fluorobenzonitrile, NaH, DMF, 80℃; (ii) 3-(R)-aminopiperidine, $NaHCO_3$, EtOH, 120℃.

The synthesis of compounds **12a-j** are outlined in Scheme 4. **11a-j** were obtained by Suzuki reaction with various phenylboric acid respectively from the bromide **10**, which were obtained by protection with Di-tert-butyl pyrocarbonate from compound **9a**. The final compounds **12a-j**, were obtained by de-protection with HCl/MeOH solution from **11a-j**.



Scheme 4. Synthesis of compounds **12a-j**.

Reagents: (i) Boc_2O, TEA, DCM, rt; (ii) Arylboronic acid, dppf.$PdCl_2$, K_2CO_3, 1,4-dioxane/H_2O, 100℃; (iii) HCl/MeOH, rt.

3. Results and discussion

*3.1. Rapid generation of the lead compound **114***

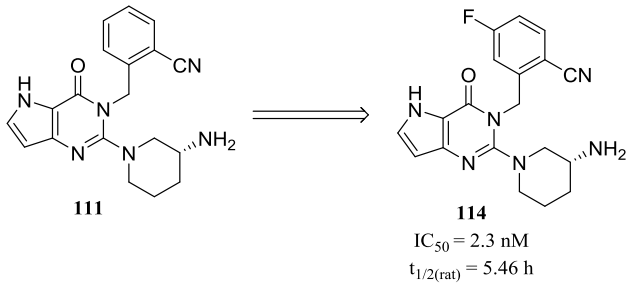


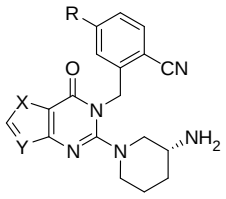
Figure 2. Rapid generation of lead compound **114**.

In our approach to enrich the alternatives of glucose-lowering agents, several DPP-4 inhibitors bearing different scaffolds or pharmacophores have been identified based on various medicinal chemistry strategies

[14-17]. Pyrrolopyrimidine scaffold was firstly being considered in this successive exploration of long-acting DPP-4 inhibitor due to its favorable PK property within our chemotypes, represented by compound **111** (Fig. 2) [14]. Tentative variation on cyanobenzyl group and heterocyclic ring were carried out on a small scale (Table 1). Reviewing the discovery of Trelagliptin from Aloglitpin, simple 5-fluoro substitution on the cyanobenzyl group resulted in a much prolonged *in vivo* DPP-4 inhibition. Despite without illustration of fluorine atom's contribution to long-acting property, several researches have discussed the SAR on benzyl group of xanthine based scaffolds. Consistently, 2- cyano, 5- fluoro- substitution provided with the best potency [18, 19]. DPP-4 crystal structure with trelagliptin revealed that the fluorine atom laid in the S1 pocket and have potential Van der Waals force with Tryp659, Tyr631, and Val656. Besides, electrical attraction between fluorine (partial negative) and ligand or protein might contributed to the increase of DPP-4 activity as well [7].

As to our small scale variation listed in Table 1, 5-fluoro substitution on cyanobenzyl group in pyrrolopyrimidine scaffold were of the better activity (compound **114-117** vs compound **112-113**), consistently with the published result. Substitution with large steric hindrance (compound **112**) or partial positive charge (compound **113**) resulted in a remarkable decrease in potency. In addition, increase the number of hetero atoms didn't improve the potency in the same direction (compound **116-117**). Nanomole level *in vitro* activity distinguished the pyrrolopyrimidine scaffold compounds **114** and **115**. Among them, compound **114** was selected to be the lead in view of close distance between the hetero atom of pyrrole ring and the caybonyl group, which might bring better absorption permeability through a hydrogen bond according to our previous experience [14].

Table 1. DPP-4 inhibitory profiles of compounds **4a-d** and **7a-b**.



No.	X	Y	R	Inhibitory IC_{50} of DPP-4 (nM)
112-7a	NH	CH		>100
113-7b	NH	CH		>100
114-4a	NH	CH	F	2.3
115-4b	CH	NH	F	2.4

116-4c	N	S	F	6.8
117-4d	NCH_3	N	F	15.3
Trelagliptin	-	-	-	1.43

Data represent means of at least two independent experiments.

*3.2 Preliminary assessment of lead compound **114***

Preliminary assessment of compound **114** were carried out subsequently, including *in vitro* activity, selectivity, and *in vivo* PK and PD evaluation. Inhibition of DPP-4 related proteases (DPP-8, DPP-9) was reported to link with multiple organ and immune system toxicities [20]. Inhibitory IC_{50} values of compound **114** against DPP-8/9 were determined to be over 300 μM, over 10 folds than that against DPP-4. And enzymatic kinetic assay indicated that compound **114** inhibited DPP-4 via a reversible non-covalent mechanism with a Ki of 2.9 nM, the same with Trelagliptin [7]. Difference laid in the specific inhibitory mode in our assay, that compound **114** inhibited DPP-4 in a mixture mode while Trelagliptin inhibited in a substrate-competitive way (Fig. 3A). The hepatic stability was evaluated in the pooled liver microsome of different species. Though species variation existed, compound **114** generally bore a good in vitro metabolic stability as expected in the pyrrolopyrimidine scaffold. The good performance in the human liver microsome indicated a probably better PK profile in human (Fig 3B, Table 3). In the PK experiment, we were pleased to see that compound **114** had a similar oral half-life than Trelagliptin ($t_{1/2(rat)}$: 5.46 h vs 5.36 h, Fig. 3C, Table 4). Accordingly, compound **114** displayed similar DPP-4 inhibition with Trelagliptin in a 52 hours PD tests, both in mice and rat (Fig. 3D, Table 5).

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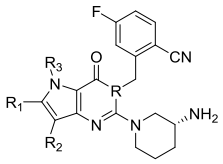
Figure 3. Preliminary evaluation of compound **114**. A. DPP-4 enzymatic kinetic assay. B. Metabolic stability in pooled liver microsome. C. Concentration- time curves in PK study. D. Blood DPP-4 inhibitory-time cures in rat and mouse.

*3.3. Further optimization and SAR study of compound **114**.*

Xanthine based DPP-4 inhibitors were mainly discovered through structural based design. The active function sites interacted with DPP-4 were generally unambiguous and nearly identical, no matter in the co-complex crystal studies of Aloglitpin and Trelagliptin, or the docking prediction of our previous study [7, 17, 21]. Aminopiperidine forms a salt bridge to the Glu205 and Glu206. Cyanobenzyl with or without F-substitution lies in the S1 pocket formed by Val656, Tyr631, Trp659, Tyr666, and Val711 and interacted with Arg125. Carbonyl group and the backbone NH of Tyr631 constitutes an important hydrogen bond which is critical for the activity. The unsaturated

scaffold conjugates π-stacking with Tyr547. Thus the pyrrolopyrimidine scaffold is supposed to be open to the structure variation, especially on the fused heterocycle.

Table 2. DPP-4 inhibitory profiles of compounds **118-133**.



No.	R_1	R_2	R_3	Inhibitory IC_{50} of DPP-4 (nM)	No.	R_1	R_2	R_3	Inhibitory IC_{50} of DPP-4 (nM)
118-9a	H	Br	H	0.79	**126-12c**	H		H	0.54
119-9b	Br	Br	H	1.67	**127-12d**	H		H	0.72
120-9c	H	F	H	0.77	**128-12e**	H		H	1.58
121-9d	F	F	H	1.25	**129-12f**	H		H	0.61
122-9e	H	H		18.0	**130-12g**	H		H	1.69
123-9f	H	H		26.6	**131-12h**	H		H	1.61
124-12a	H		H	0.76	**132-12i**	H		H	1.45
125-12b	H		H	0.54	**133-12 j**	H		H	0.77

Data represent means of at least two independent experiments.

Initially we attempted to evaluate the effect of substitutions on N-, α-, β- of pyrrole ring on the DPP-4 activity (compound **118**-**123**). Substitutions were generally tolerable except for the N-substitution which decreased the activity into double-digit IC_{50} value in compound **122** and **123**. Single β-substitution or α, β-substitution kept a well DPP-4 affinity with β-substitution a bit better (compound **118**/**120** v.s. compound **119**/**121**). Hence, extended variation on β-substitution was undertaken and lead to compounds **124** to **133**. It's pleased to see that β-position could tolerate a range of wide variation with good activity at single-digit nanomole level despite of steric hindrance or polarity of substituent group. All the β-substituted compounds were delivered to examine their metabolic stability in both rat liver microsome (RLM) and human liver microsome (HLM). With two compounds

with poorer stability in HLM than that in RLM (compound **125**, **130**), the rest compounds in Table 3 were not supposed to suffer from severe hepatic clearance and put into *in vivo* pharmacokinetic study. During the course of PK screening, compound **124** displayed longer half-life than Trelagliptin in rat and was chosen to receive the *in vivo* PD evaluation (Table 4& 5).

why not choose 128,131 for next test?

Table 3. *In vitro* metabolic stability of β-substituted compounds in pooled liver microsome.

No.	Calculated $t_{1/2}$ (min)		No.	Calculated $t_{1/2}$ (min)	
	RLM	HLM		RLM	HLM
114	45.2	>200	**128**	197.1	>200
118	37.5	132.7	**129**	98.9	>200
120	50.3	98.2	**130**	>200	43.3
124	47.0	160.0	**131**	>200	>200
125	36.52	35.1	**132**	166.9	>200
127	72.3	180.8	**133**	95.2	>200

RLM: pooled rat liver microsome. HLM: human pooled liver microsome. Data represent as the average of three duplicates.

*3.4. PK/PD evaluation of compound **124**.*

Compound **124** is a potent DPP-4 inhibitor with IC_{50} value of 0.76 nM and good selectivity against DPP-8/9. The hepatic stability is generally acceptable with calculated half-life of 47 min and 160 min in RLM and HLM incubation respectively. Moreover, in the pharmacokinetic study, compound **124** showed a prolonged half-life compared with Trelagliptin in a same batch ($t_{1/2}$: 7.89 h v.s. 5.13 h, Fig. 4A, Table 4). The 52 hours' *in vivo* PD test in rat and mouse both demonstrated that compound **124** had a similar or slightly better DPP-4 inhibition than Trelagliptin, both in the total DPP-4 inhibition and the point DPP-4 inhibition of last sampling at 52 hours (Fig. 4B, Table 5).

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Figure 4. *In vivo* PK and PD evaluation of compound 124. A. Concentration- time curves in PK study. B. Blood DPP-4 inhibitory-time cures in rat and mouse.

Table 4. Pharmacokinetic profiles of compound **114**, **124**, and Trelagliptin.

	oral $T_{1/2}$ (h)	T_{max} (h)	$poAUC_{0-t}$ ($\mu g \cdot h \cdot mL^{-1}$)	CLp ($L \cdot h^{-1} \cdot kg^{-1}$)	$V_{Z/F}$ ($L \cdot kg^{-1}$)	F%
114	5.46 ±3.74	0.14 ±0.10	1.88 ±0.06	13.3 ±0.41	105.5 ±74.2	10.6
124	7.89 ±5.41	2.33 ±0.57	2.92 ±0.56	8.80 ±1.81	103.5 ±80.2	33.4
Trelaglitpin	5.13 ±1.74	0.70 ±0.14	6.82 ±1.96	7.66 ±2.82	48.2 ±20.15	46.9

i.v., intravenous injection; p.o. oral administration. Dose: 5 and 25 mg/kg for i.v. and p.o. respectively.

3.5. Potential factors contributed to the long-acting of pyrrolopyrimine scaffold based DPP-4 inhibitors.

Patient adherence is very important to the progress of the chronic diseases, especially for the T2D. Although the advantage is under controversy, the development of long-acting DPP-4 inhibitors should still be on the way, at least for those first diagnosed T2D patients who had metformin contradiction and invalid lifestyle alteration [13]. Firstly, we hypothesized that a slow-binding or dissociation between compound and DPP-4 enzyme might be a possible mechanism of long-acting. Preliminary drug-target binding kinetics evaluation were conducted on typical once-weekly drugs Omarigliptin, Trelagliptin, and regular once-dailiy Sitagliptin and Aloglitpin. However, the dissociation rate constant (k_{off}) data between these two types of drugs did have a distance in our research, but not distinguishable enough. This conclusion also coincided with the later published evaluations of Trelaglitpin [7, 22]. Thus we turned our attention back to the traditional PK-PD strategy. During the course of optimization, it's quite challenging to determine the specific PK parameters to improve. In our initial pharmacokinetic comparison between once-weekly and once-daily DPP-4 inhibitors in rat, we noticed a longer half-life and a lower clearance in once-weekly Trelaglitpin and Omarigliptin. But the differences of half-life and clearance weren't capable enough to sustain the week-long efficacy. Then we had to mainly rely on the *in vivo* measures to screen these batch of compounds. Considering that β-substituted pyrrolopyrimidine compounds kept an excellent in vitro DPP-4 inhibitory activity, we delivered the compounds whose half-life were similar or better than Trelagliptin to the *in vivo* PD tests and discovered compound **114** and **124**.

Reviewing the research and development of long-acting pyrrolopyrimidine based DPP-4 inhibitors, we affirmed optimization of PK profile is a feasible way to obtain long-acting compounds first of all. Half-life is an important parameter. But it's hard to take it as a decisive factor based on the available data. And it's not a fine parameter for high throughput screening, which couldn't be easily correlated with *in vitro* indicators like hepatic stability. As oral administrated drugs, bioavailability seemed not to be a determinant for sustained efficacy (Table 4). Secondly, it's generally believed that the glycaemic benefits of DPP-4 inhibitors are related to the residual inhibition of the enzyme at the end of the dosing interval [13]. In the 52 hours' PD evaluations, compound **114** and

124 both had a stronger inhibition than Trelagliptin at the last sampling point except for the 10 mg/kg dosing group in rat (Table 5). Hence, long duration of *in vitro* DPP-4 inhibition test might be a probable way to screen long-acting compounds after establishment of a reliable long duration *in vitro* reaction system. Finally, comprehensive evaluation of Trelagliptin reminded that the potency partially contributed to the *in vivo* efficacy due to a stronger inhibition at a lower concentration. For this concept, compound **114** and **124** displayed a lower and a better IC_{50} values than Trelagliptin in our assay, yet a similar *in vivo* efficacy. Though the three compounds were at the same level of potency, the contribution of DPP-4 affinity couldn't be drawn solidly. We believed that the higher potency is meaningful for the sustained long efficacy.

Table 5. Pharmacodynamic evaluation of compounds **114** and **124**.

	DPP-4 inhibition (%)	rat		mouse	
		3 mg/kg	10 mg/kg	3 mg/kg	10 mg/kg
In total	**114**	28.71%	36.57%	21.76%	20.12%
	124	28.31%	37.83%	10.0%	17.25%
	Trelaglitpin	21.75%	39.46%	2.5%	12.13%
At 52 hours	**114**	14.32%	38.54%	23.06%	17.94%
	124	13.25%	46.56%	11.35%	15.73%
	Trelaglitpin	2.14%	72.63%	-8.33%	1.03%

4. Conclusion

In the continuation of our discovery of long-acting DPP-4 inhibitors, previously reported pyrrolopyrimidine scaffold based compound **111** was chosen to be the hit compound due to pharmacokinetic superiority. Inspired by the discovery of Trelagliptin, tentative variation on pyrrole ring gave the lead compound **114** (IC_{50} = 2.3 nM, $t_{1/2(rat)}$ = 5.46 h). With the consideration of binding mode between xanthine based compounds and DPP-4, extensive lead optimization was conducted. β-substitution on pyrrole ring was determined to be a good position open to wild variation. Meanwhile excellent DPP-4 inhibitory activity was kept of β-substituted compounds represented by compound **124** (IC_{50} = 0.76 nM, $t_{1/2(rat)}$ = 7.89 h). *In vivo* pharmacodynamics study demonstrated a similar or slightly better sustained efficacy compared with Trelagliptin. Reviewing the development course, improved pharmacokinetic profile was important to prolong the efficacy of pyrrolopyrimidine or xanthine based DPP-4 inhibitors, especially for the half-life or the DPP-4 inhibition after a long duration. Based on this

preliminary data, compound **114** and **124** will be delivered into more comprehensive evaluation in the due course. And in depth dose-response relation research will be carried out to evaluate the action nature of these compounds.

5. Experimental section

5.1. Chemistry

All commercially available compounds and solvents were of reagent grade and were used without further treatment unless otherwise noted. Reactions were monitored by TLC using Qing Dao Hai Yang GF254 silica gel plates (5 x 10 cm); zones were detected visually under ultraviolet irradiation (254 nm) and by spraying with an ethanol solution of 2,4-DNP or ninhydrin or by fuming with an iodine steam. Silica gel column chromatography was performed on silica gel (200-300 mesh) from Qing Dao Hai Yang. NMR spectra were recorded on a Bruker NMR AVANCE 400 (400 MHz) or a Bruker NMR AVANCE 500 (500 MHz). Chemical shifts (δ) were recorded in ppm and coupling constants (J) in hertz (Hz). Splitting patterns describe apparent multiplicities and are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) or br (broad). MS data were measured on an Agilent MSD-1200 ESI-MS system.

*5.1.1. 2-((2-chloro-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile (**3a**)*

NaH (60% in oil, 0.46 g, 11.5 mmol) was added to a stirred solution of **2a'** (2.70 g, 10.0 mmol) in DME (40 mL) and DMF (10 mL) at 0℃. Twenty minutes later, LiBr (1.74 g, 20.0 mmol) was added, and the mixture was allowed to warm to room temperature. After 15 min, 2-(bromomethyl)-4-fluorobenzonitrile (2.46 g, 11.5 mmol) was then added, and the mixture was heated at 65℃ overnight. After cooling, the mixture was poured into water (250 mL) and extracted with ethyl acetate (250*3 mL). The organic layer was combined and dried with anhydrous sodium sulphate, and filtered. The resulting filtrate was concentrated in vacuo and then purified by flash chromatography to yield the title compound **3a** as white powder (2.27 g, yield 75.2%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.28 (s, 1H), 7.76-7.23 (m, 1H) , 7.40-7.39 (m, 1H), 7.14-7.09 (m, 1H), 6.85-6.82 (m, 1H), 6.55-6.54 (m, 1H), 5.77 (s, 2H); ESI-MS calculated for ($C_{14}H_8ClFN_4O$) $[M + H]^+$, 302.04, found 303.0.

*5.1.2.(R)-2-((2-(3-aminopiperidin-1-yl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzo nitrile (**4a**)*

A mixture of **3a** (3.03 g, 10.0 mmol), 3-(R)-aminopiperidine dihydrochloride (2.07 g, 12.0 mmol) and $NaHCO_3$ (2.10 g, 25.0 mmol) in a sealed tube containing 30 mL of ethanol was heated at 120℃ overnight. The reaction mixture was subsequently cooled to room temperature and filtered. The resulting filtrate was concentrated

in vacuo and then purified by flash chromatography to yield compound **4a** as white foam (2.33 g, yield 63.5%). ^{1}H NMR (400 MHz, MeOD) δ: 7.85-7.81 (m, 1H), 7.37 (s, 1H), 7.22-7.18 (m, 1H), 6.76-6.74 (d, *J* = 9.6 Hz, 1H), 6.40 (s, 1H), 5.60 (s, 2H), 3.26-3.23 (m, 1H), 3.06-3.03 (m, 1H), 2.86-2.75 (m, 2H), 2.66-2.61 (m, 1H), 1.96-1.94 (m, 1H), 1.78-1.75 (m, 1H), 1.63-1.60 (m, 1H), 1.29-1.24 (m, 1H); ^{13}C NMR (125 MHz, MeOD) δ: 166.32, 164.29, 155.46, 154.27, 145.45, 143.39, 135.58, 128.52, 116.14, 114.94, 114.05, 106.64, 102.45, 58.26, 53.32, 51.33, 44.85, 32.52, 22.84; ESI-MS calculated for ($C_{19}H_{19}FN_6O$) $[M + H]^+$, 366.16, found 367.1.

*5.1.3.(R)-2-((2-(3-aminopiperidin-1-yl)-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-3-yl)methyl)-4-fluorobenzo nitrile (**4b**).*

Compound **4b** was prepared in a manner identical to that described for **4a** as yellow powder. Yield: 53.3%.^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.65 (s, 1H), 7.64-7.63 (m, 1H), 7.45-7.42 (m, 1H), 7.31-7.29 (m, 1H), 7.03-7.01 (d, *J* = 6.4 Hz, 1H), 6.82-6.81 (d, *J* = 2.4 Hz, 1H), 6.64-6.63 (d, *J* = 2.4 Hz, 1H), 5.59-5.52 (dd, *J* = 4.4 Hz, *J* = 17.2 Hz, 2H), 3.16-3.14 (m, 1H), 2.98-2.93 (m, 2H), 2.75-2.71 (m, 1H), 2.65-2.61 (m, 1H), 1.92 (s, 2H), 1.74-1.71 (m, 1H), 1.64-1.59 (m, 1H), 1.24-1.22 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 160.28, 156.33, 147.09, 141.79, 133.16, 132.79, 127.47, 126.87, 119.28, 117.36, 110.63, 104.45, 103.78, 58.48, 51.70, 47.35, 45.71, 33.32, 23.06; ESI-MS calculated for ($C_{19}H_{19}FN_6O$) $[M + H]^+$, 366.16, found 367.1.

*5.1.4.(R)-2-((5-(3-aminopiperidin-1-yl)-7-oxothiazolo[5,4-d]pyrimidin-6(7H)-yl)methyl)-4-fluorobenzonitrile (**4c**).*

Compound **4c** was prepared in a manner identical to that described for **4a** as yellow oil. Yield: 36.4%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.48 (s, 1H), 7.68-7.67 (m, 1H), 7.43-7.41 (m, 1H), 7.09 (s, 1H), 5.74 (s, 2H), 4.41-4.37 (m, 1H), 3.79-3.63 (m, 2H), 3.09-3.03 (m, 1H), 2.87 (s, 1H), 1.97 (s, 2H), 1.83-1.76 (m, 1H), 1.49-1.47 (m, 1H), 1.24-1.22 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 167.08, 163.47, 156.81, 152.55, 147.56, 143.78, 133.64, 119.05, 115.88, 114.63, 114.25, 106.82, 50.90, 50.13, 42.75, 37.10, 36.43, 23.07; ESI-MS calculated for ($C_{18}H_{17}FN_6OS$) $[M + H]^+$, 384.12, found 385.1.

*5.1.5.(R)-2-((2-(3-aminopiperidin-1-yl)-7-methyl-6-oxo-6,7-dihydro-1H-purin-1-yl)methyl)-4-fluorobenzonitrile (**4d**).*

Compound **4d** was prepared in a manner identical to that described for **4a** as yellow oil. Yield: 38.3%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.04 (s, 1H), 7.81 (s, 1H), 7.39 (s, 1H), 7.11-7.04 (m, 1H), 5.49 (s, 2H), 4.73 (s, 3H), 4.30-4.17 (m, 1H), 3.43 (s, 1H), 2.96 (s, 2H), 2.76 (s, 1H), 1.99 (s, 2H), 1.63 (s, 1H), 1.41 (m, 1H), 1.22 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 167.06, 163.12, 156.84, 152.09, 147.54, 144.43, 133.68, 115.84, 115.51, 114.28,

106.85, 50.92, 50.12, 42.72, 36.48, 33.34, 23.07; ESI-MS calculated for ($C_{19}H_{20}FN_7O$) $[M + H]^+$, 381.17, found 382.2.

*5.1.6. 2-((2-chloro-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile (**5**)*

Di-tert-butyl dicarbonate (2.62 g, 12.0 mmol) was dropped to a mixture of **4a** (3.66 g, 10.0 mmol) and triethylamine (1.11 g, 11.0 mmol) in a round flask containing 60 mL of dichloromethane in room temperature, and the solution was stirred in room temperature overnight. The reaction mixture was poured into water (100 mL) and extracted with ethyl acetate (100*3 mL). The organic layer was combined and dried with anhydrous sodium sulphate, and filtered. The resulting filtrate was concentrated in vacuo and then purified by flash chromatography to yield the title compound **5** as white foam (4.21 g, yield 90.4%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.97 (s, 1H), 7.98-7.94 (m, 1H), 7.38-7.37 (m, 1H), 7.35-7.29 (m, 1H), 6.86-6.79 (m, 2H), 6.32-6.31 (m, 1H), 5.48-5.42 (m, 2H), 3.39-3.37 (m, 1H), 3.09-2.99 (m, 2H), 2.66-2.54 (m, 2H), 1.77 (m, 1H), 1.75 (m, 1H), 1.72-1.68 (m, 1H), 1.53-1.47 (m, 1H), 1.36-1.35 (m, 1H), 1.34 (s, 9H); ESI-MS calculated for ($C_{24}H_{27}FN_6O_3$) $[M + H]^+$, 466.21, found 467.1.

*5.1.7. (R)-tert-butyl 1-(3-(2-cyano-5-morpholinobenzyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-2-yl)piperidin-3-ylcarbamate (**6a**)*

A mixture of **5** (2.33 g, 5.0 mmol), morpholine (1.31 g, 15.0 mmol), CuI (2.86 g, 15.0 mmol) and K_2CO_3 (1.38 g, 10.0 mmol) in a sealed tube containing 30 mL of methylsulfinylmethane was heated at 120℃ in Ar_2 atmosphere overnight. The reaction mixture was subsequently cooled to room temperature and poured into water (80 mL) and extracted with ethyl acetate (80*3 mL). The organic layer was combined and dried with anhydrous sodium sulphate, and filtered. The resulting filtrate was concentrated in vacuo and then purified by flash chromatography to yield the title compound **6a** as light-yellow solid (1.26 g, yield 47.4%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.59 (s, 1H), 7.52-7.50 (m, 1H), 7.34-7.32 (m, 1H), 6.73-6.70 (m, 1H), 6.44-6.43 (m, 1H), 6.38 (s, 1H), 5.53 (s, 2H), 4.91 (s, 1H), 3.77 (s, 1H), 3.74-3.72 (m, 4H), 3.40-3.37 (m, 1H), 3.11-3.09(m, 4H), 3.01 (s, 1H), 2.88-2.86 (m, 2H), 1.82 (s, 2H), 1.70 (s, 1H), 1.48 (m, 1H), 1.42 (s, 9H); ESI-MS calculated for ($C_{28}H_{35}N_7O_4$) $[M + H]^+$, 533.28, found 534.3.

*5.1.8.(R)-2-((2-(3-aminopiperidin-1-yl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-morpholino benzonitrile hydrochloride (**7a**)*

Compound **6a** (1.20 g, 2.2 mmol) was dissolved in 30mL saturated HCl/MeOH solution and stirred overnight at room temperature. The reaction solution was concentrated in vacuo to give a yellow solid. The solid was suspended in 50mL aether and filtered. The filter cake was washed by aether and dried to yield the title compound **7a** as light-yellow solid (0.91 g, yield 85.8%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.05-11.98 (m, 1H), 8.26-8.14 (m, 3H), 7.59-7.57 (m, 1H), 7.37-7.36 (m, 1H), 6.93-6.91 (m, 1H), 6.53-6-51 (m, 1H), 6.32 (s, 1H), 5.39-5.26 (m, 2H), 3.46-3.42 (m, 4H), 3.35-3.26 (m, 1H), 3.13-3.12 (m, 2H), 3.03-3.01 (m, 4H), 2.96-2.93 (m, 2H), 2.67 (m, 1H), 1.98 (s, 1H), 1.75-1.73 (m, 1H), 1.54-1.51 (m, 2H); ^{13}C NMR (125 MHz, MeOD) δ: 155.41, 153.64, 152.64, 140.54, 134.33, 133.09, 131.18, 129.75, 118.24, 115.31, 114.55, 113.31, 97.69, 66.08, 51.88, 50.95, 50.08, 46.61, 29.74, 27.35, 22.13; ESI-MS calculated for ($C_{23}H_{27}N_7O_2$) $[M + H]^+$, 433.22, found 434.2.

*5.1.9.(R)-2-((2-(3-aminopiperidin-1-yl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-(dimethylamino)benzonitrile (**7b**).*

Compound **7b** was prepared in a manner identical to that described for **7a** as yellow solid. Yield (two steps): 35.3%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 11.08 (s, 1H), 7.44-7.42 (d, *J* = 8.8 Hz, 1H), 7.25-7.24 (d, *J* = 2.8 Hz, 1H), 6.50-6.47 (dd, *J* = 8.8 Hz, *J* = 2.4 Hz, 1H), 6.40-6.39 (d, *J* = 2.8 Hz, 1H), 6.40-6.39 (d, *J* = 2.8 Hz, 1H), 6.07-6.06 (d, *J* = 2.4 Hz, 1H), 5.55-5.46 (m, 2H), 3.24-3.21 (m, 1H), 3.06-3.02 (m, 1H), 2.99 (s, 2H), 2.88 (s, 1H), 2.82 (s, 6H), 2.77-2.72 (m, 2H), 1.92-1.89 (m, 1H), 1.77-1.75 (m, 1H), 1.66-1.58 (m, 1H), 1.35-1.25 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$+MeOD) δ: 156.14, 154.29, 152.82, 143.33, 142.39, 134.05, 128.51, 119.25, 115.17, 110.27, 108.32, 103.13, 95.33, 56.85, 51.78, 46.95, 45.74, 39.62, 29.60, 22.59; ESI-MS calculated for ($C_{21}H_{25}N_7O$) $[M + H]^+$, 391.21, found 392.2.

*5.1.10.(R)-2-((2-(3-aminopiperidin-1-yl)-7-bromo-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile hydrochloride (**9a**).*

Compound **9a** was prepared in a manner identical to that described for **4a** as yellow solid with one hydrochloride. Yield (last step): 63.2%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.43 (s, 1H), 8.23 (s, 3H), 7.97-7.93 (m, 1H), 7.58-7.57 (d, *J* = 3.2 Hz, 1H), 7.35-7.30 (m, 1H), 7.00-6.98 (m, 1H), 5.52-5.33 (dd, *J* = 16.0 Hz, *J* = 63.6 Hz, 2H), 3.45-3.38 (m, 1H), 3.26 (m, 1H), 3.02 (s, 2H), 2.74 (s, 1H), 1.98-1.97 (m, 1H), 1.80 (s, 1H), 1.57 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$+MeOD) δ: 165.96, 163.94, 154.58, 145.87, 140.13, 136.39, 128.32, 117.10, 115.96, 115.64, 115.37, 106.99, 90.12, 52.70, 52.04, 46.86, 45.91, 27.65, 22.15; ESI-MS calculated for ($C_{19}H_{18}BrFN_6O$) $[M + H]^+$, 444.07, 446.07, found 445.1, 447.1.

5.1.11.(R)-2-((2-(3-aminopiperidin-1-yl)-6,7-dibromo-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)

*-4-fluorobenzonitrile hydrochloride (**9b**).*

Compound **9b** was prepared in a manner identical to that described for **4a** as yellow solid with one hydrochloride. Yield (last step): 66.2%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 13.32 (s, 1H), 8.36 (s, 3H), 7.92 (s, 1H), 7.30 (s, 1H), 7.02-7.01 (m, 1H), 7.00-6.98 (m, 1H), 5.49-5.30 (dd, *J* = 14.4 Hz, *J* = 62.4 Hz, 2H), 3.38 (s, 1H), 3.23 (s, 1H), 3.00-2.98 (m, 2H), 2.73 (s, 1H), 1.97 (s, 1H), 1.79 (s, 1H), 1.56 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$+MeOD) δ: 165.90, 163.88, 155.15, 153.64, 145.54, 140.73, 136.41, 117.01, 116.71, 115.51, 115.33, 114.46, 106.89, 93.25, 52.56, 51.96, 46.81, 46.16, 27.67, 22.14; ESI-MS calculated for ($C_{19}H_{17}Br_2FN_6O$) $[M + H]^+$, 523.98, found 525.0.

*5.1.12.(R)-2-((2-(3-aminopiperidin-1-yl)-7-fluoro-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile hydrochloride (**9c**).*

Compound **9c** was prepared in a manner identical to that described for **4a** as yellow solid with one hydrochloride. Yield (last step): 56.2%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.35-12.34 (d, *J* = 2.4 Hz, 1H), 8.23 (s, 3H), 7.97-7.94 (m, 1H), 7.57-7.56 (d, *J* = 3.2 Hz, 1H), 7.36-7.31 (m, 1H), 7.07-6.97 (m, 1H), 5.52-5.33 (m, 2H), 3.41-3.37 (m, 1H), 3.25 (s, 1H), 3.07-3.03 (m, 2H), 2.74 (s, 1H), 2.00-1.98 (m, 1H), 1.80 (s, 1H), 1.59 (s, 2H); ^{13}C NMR (125 MHz, MeOD) δ: 166.57, 164.14, 154.99, 154.01, 144.88, 138.39, 135.67, 125.82, 116.31, 115.34, 114.82, 106.71, 105.12, 51.89, 51.19, 45.67, 33.31, 27.54, 21.29; ESI-MS calculated for ($C_{19}H_{18}F_2N_6O$) $[M + H]^+$, 384.15, found 385.1.

*5.1.13.(R)-2-((2-(3-aminopiperidin-1-yl)-6,7-difluoro-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile (**9d**).*

Compound **9d** was prepared in a manner identical to that described for **4a** as white foam. Yield (last step): 58.9%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.68-7.66 (m, 1H), 7.06-7.02 (m, 1H), 6.66-6.63 (m, 1H), 5.57 (s, 2H), 3.63 (s, 2H), 3.24-3.22 (m, 1H), 3.06-2.99 (m, 2H), 2.90-2.84 (m, 1H), 2.78-2.73 (m, 1H), 1.96-1.93 (m, 1H), 1.80-1.77 (m, 1H), 1.67-1.58 (m, 1H), 1.33-1.25 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 166.34, 163.57, 154.98, 154.32, 145.36, 139.05, 135.90, 126.45, 116.97, 115.42, 114.21, 106.98, 105.77, 52.47, 51.72, 46.01, 34.29, 27.56, 21.39; ESI-MS calculated for ($C_{19}H_{17}F_3N_6O$) $[M + H]^+$, 402.14, found 403.1.

*5.1.14.2-((2-chloro-5-(methylsulfonyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile (**8e**)*

Methanesulfonyl chloride (2.28 g, 20.0 mmol) was dropped to a mixture of **3a** (3.03 g, 10.0 mmol) and triethylamine (2.23 g, 22.0 mmol) in a round flask containing 60 mL of N,N-dimethylformamide in 0℃, and the

solution was stirred in room temperature overnight. The reaction mixture was poured into water (100 mL) and extracted with ethyl acetate (100*3 mL). The organic layer was combined and washed with saturated sodium carbonate aqueous solution, water, and saturated salt solution successively, and dried with anhydrous sodium sulphate, and filtered. The resulting filtrate was concentrated in vacuo and then purified by flash chromatography to yield the title compound **8e** as white solid (3.26 g, yield 85.7%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.06-8.03 (m, 1H), 7.94-7.93 (d, *J* = 2.8 Hz, 1H), 7.43-7.39 (m, 1H), 7.31-7.29 (d, *J* = 9.6 Hz, 1H), 6.73 (s, 1H), 5.60 (s, 2H), 3.85 (s, 3H); ESI-MS calculated for ($C_{15}H_{10}ClFN_4O_3S$) $[M + H]^+$, 380.01, found 381.0.

*5.1.15.(R)-2-((2-(3-aminopiperidin-1-yl)-5-(methylsulfonyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile (**9e**).*

Compound **9e** was prepared in a manner identical to that described for **4a** as white solid. Yield: 67.5%. ^{1}H NMR (400 MHz, MeOD) δ: 7.85-7.83 (m, 1H), 7.80-7.79 (d, *J* = 3.2 Hz, 1H), 7.24-7.20 (m, 1H), 6.98-6.95 (d, *J* = 9.6 Hz, 1H), 6.55-6.54 (d, *J* = 2.8 Hz, 1H), 5.78 (s, 2H), 3.72 (s, 3H), 3.38-3.33 (m, 1H), 3.19 (s, 1H), 2.94-2.89 (m, 1H), 2.86-2.80 (m, 1H), 2.70-2.65 (m, 1H), 1.99-1.97 (m, 1H), 1.80-1.77 (m, 1H), 1.70-1.61 (m, 1H), 1.33-1.25 (m, 1H); ^{13}C NMR (125 MHz, MeOD) δ: 166.31, 164.28, 155.44, 154.26, 145.44, 143.38, 135.59, 128.53, 116.15, 115.10, 114.07, 106.61, 102.45, 58.12, 51.34, 47.03, 32.68, 32.23, 23.18; ESI-MS calculated for ($C_{20}H_{21}FN_6O_3S$) $[M + H]^+$, 444.14, found 445.1.

*5.1.16.(R)-2,5'-(2-(3-aminopiperidin-1-yl)-4-oxo-3H-pyrrolo[3,2-d]pyrimidine-3,5(4H)-diyl)bis(methylene)bis(4-fluorobenzonitrile) hydrochloride (**9f**).*

Compound **9f** was prepared in a manner identical to that described for **9e** as white solid with one hydrochloride. Yield (last step): 58.9%. ^{1}H NMR (400 MHz, MeOD) δ: 7.69-7.67 (m, 1H), 7.65-7.64 (m, 1H), 7.54 (s, 1H), 7.20-7.18 (m, 1H), 7.16-7.10 (m, 2H), 6.49 (s, 2H), 5.66 (s, 2H), 5.39-5.31 (m, 2H), 3.82-3.81 (m, 1H), 3.58 (s, 1H), 3.46-3.44 (m, 1H), 3.32-3.30 (m, 1H), 3.19 (s, 1H), 2.19-2.17 (m, 1H), 1.96-1.93 (m, 1H), 1.86-1.85 (m, 1H), 1.70-1.69 (m, 1H); ^{13}C NMR (125 MHz, MeOD) δ: 166.10, 165.82, 164.07, 163.78, 155.20, 153.18, 144.73, 143.33, 135.54, 133.90, 117.59, 116.32, 115.92, 115.75, 114.67, 114.48, 114.09, 106.87, 106.42, 99.83, 51.93, 51.02, 49.40, 48.49, 46.58, 27.35, 21.97; ESI-MS calculated for ($C_{27}H_{23}F_2N_7O$) $[M + H]^+$, 499.19, found 500.2.

*5.1.17. (R)-tert-butyl 1-(7-bromo-3-(2-cyano-5-fluorobenzyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-2-yl)piperidin-3-ylcarbamate (**10**).*

Compound **10** was prepared from **9a** in a manner identical to that described for **5** as yellow solid. Yield: 88.7%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.87 (s, 1H), 7.71-7.68 (m, 1H), 7.34-7.33 (d, *J* = 2.8 Hz, 1H), 7.06-7.02 (m, 1H), 6.69-6.67 (d, *J* = 7.6 Hz, 1H), 5.56 (s, 2H), 5.08 (s, 1H), 3.77 (s, 1H), 3.37-3.33 (m, 1H), 3.09 (s, 1H), 3.03-2.96 (m, 2H), 1.84 (s, 2H), 1.56-1.53 (m, 1H), 1.41 (s, 9H), 1.27-1.26 (m, 1H); ESI-MS calculated for ($C_{24}H_{26}BrFN_6O_3$) $[M + H]^+$, 544.12, 546.12, found 545.1, 547.1.

*5.1.18. (R)-tert-butyl 1-(3-(2-cyano-5-fluorobenzyl)-4-oxo-7-(thiophen-3-yl)-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-2-yl)piperidin-3-ylcarbamate (**11a**).*

A mixture of **10** (1.09 g, 2.0 mmol), thiophen-3-ylboronic acid (0.31 g, 2.4 mmol), 1,1'-Bis(diphenylphosphino)ferrocene-palladium(II)dichloride dichloromethane complex ($Pd(dppf)Cl_2$.DCM, 0.10 g, 0.12 mmol) and K_2CO_3 (0.69 g, 5.0 mmol) in a sealed tube containing 10 mL of 1,4-dioxane and 2mL water was heated at 100℃ in Ar_2 atmosphere overnight. The reaction mixture was subsequently cooled to room temperature and poured into water (80 mL) and extracted with ethyl acetate (100*3 mL). The organic layer was combined and washed with saturated sodium carbonate aqueous solution, water, and saturated salt solution successively, and dried with anhydrous sodium sulphate, and filtered. The resulting filtrate was concentrated in vacuo and then purified by flash chromatography to yield the title compound **11a** as white solid (0.86 g, yield 78.3%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 11.54 (s, 1H), 7.98-7.97 (d, *J* = 2.0 Hz, 1H), 7.68 (s, 1H), 7.56-7.55 (m, 2H), 7.37-7.36 (m, 1H), 7.01-6.99 (m, 1H), 6.74-6.72 (m, 1H), 5.68-5.53 (m, 2H), 4.76 (s, 1H), 3.86 (m, 1H), 3.41-3.39 (m, 1H), 3.06-3.01 (m, 2H), 2.90 (s, 1H), 1.88-1.83 (m, 2H), 1.74 (s, 1H), 1.47 (s, 1H), 1.41 (s, 9H); ESI-MS calculated for ($C_{28}H_{29}FN_6O_3S$) $[M + H]^+$, 548.20, found 549.2.

*5.1.19.(R)-2-((2-(3-aminopiperidin-1-yl)-4-oxo-7-(thiophen-3-yl)-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile hydrochloride (**12a**).*

Compound **12a** was prepared from **11a** in a manner identical to that described for **7a** as white solid. Yield: 89.1%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.13-12.12 (d, *J* = 2.8 Hz, 1H), 8.16 (s, 1H), 8.02 (d, *J* = 1.2 Hz, 1H), 8.01-7.96 (m, 1H), 7.86-7.85 (d, *J* = 2.8 Hz, 1H), 7.74-7.73 (m, 1H), 7.60-7.58 (m, 1H), 7.377.32 (m, 1H), 6.98-6.95 (m, 1H), 5.56-5.38 (m, 2H), 3.48-3.46 (m, 1H), 3.36 (s, 1H), 3.09-3.03 (m, 2H), 2.82 (s, 1H), 1.99 (s, 1H), 1.84 (s, 1H), 1.60-1.58 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d_6+MeOD) δ: 166.04, 164.02, 154.91, 154.09, 146.17, 139.64, 136.41, 134.66, 126.73, 116.23, 115.33, 112.97, 107.16, 52.85, 52.15, 46.96, 45.73, 27.84, 22.28; ESI-MS calculated for ($C_{23}H_{21}FN_6OS$) $[M + H]^+$, 448.15, found 449.1.

*5.1.20.(R)-2-((2-(3-aminopiperidin-1-yl)-4-oxo-7-(thiophen-3-yl)-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile hydrochloride (**12b**).*

Compound **12b** was prepared in a manner identical to that described for **12a** as white solid. Yield (two steps): 62.6%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.21 (s, 1H), 8.30 (s, 3H), 7.93 (s, 1H), 7.75 (s, 1H), 7.65-7.43 (m, 1H), 7.36-7.31 (m, 2H), 7.06-6.85 (m, 2H), 5.54-5.36 (m, 2H), 3.26-3.15 (m, 2H), 3.09-3.00 (m, 2H), 2.82 (s, 1H), 1.97 (s, 1H), 1.82 (s, 1H), 1.58 (s, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 166.08, 164.04, 154.86, 154.24, 146.04, 139.10, 136.48, 135.73, 127.70, 125.27, 123.66, 122.87, 117.15, 116.14, 115.35, 111.83, 107.14, 52.94, 52.13, 46.83, 45.86, 27.89, 22.24; ESI-MS calculated for ($C_{23}H_{21}FN_6OS$) $[M + H]^+$, 448.15, found 449.1.

*5.1.21.(R)-2-((2-(3-aminopiperidin-1-yl)-7-(4-fluorophenyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile hydrochloride (**12c**).*

Compound **12c** was prepared in a manner identical to that described for **12a** as white solid. Yield (two steps): 66.3%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.21 (s, 1H), 8.25 (s, 3H), 8.12 (s, 2H), 7.95 (s, 1H), 7.88 (s, 1H), 7.33 (s, 1H), 7.22-7.20 (m, 2H), 6.99-6.97 (m, 1H), 5.54-5.38 (m, 2H), 3.46-3.44 (m, 2H), 3.07-3.01 (m, 2H), 2.84 (s, 1H), 1.98 (s, 1H), 1.83 (s, 1H), 1.59 (s, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 161.88, 160.05, 154.95, 153.99, 146.19, 139.67, 136.47, 130.90, 128.04, 126.16, 117.19, 116.66, 115.75, 115.21, 107.16, 52.93, 51.98, 46.84, 45.77, 27.89, 22.13; ESI-MS calculated for ($C_{25}H_{22}F_2N_6O$) $[M + H]^+$, 460.18, found 461.2.

*5.1.22.(R)-2-((2-(3-aminopiperidin-1-yl)-7-(4-(methylsulfonyl)phenyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile hydrochloride (**12d**).*

Compound **12d** was prepared in a manner identical to that described for **12a** as white solid. Yield (two steps): 64.8%. ^{1}H NMR (400 MHz, MeOD) δ: 8.47 (s, 2H), 8.06 (s, 3H), 7.94 (s, 1H), 7.76 (s, 2H), 7.68 (s, 1H), 7.34 (s, 1H), 7.13 (s, 1H), 5.73-5.64 (m, 2H), 3.73-3.65 (m, 2H), 3.31 (s, 1H), 3.26 (s, 1H), 3.26 (s, 3H), 2.26 (s, 1H), 2.00-1.92 (m, 2H), 1.77 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6+MeOD) δ: 166.22, 164.22, 154.04, 140.50, 139.73, 137.34, 135.86, 132.42, 127.34, 127.06, 126.33, 116.73, 115.46, 114.96, 106.86, 52.60, 51.83, 47.12, 45.70, 43.41, 27.79, 22.04; ESI-MS calculated for ($C_{26}H_{25}FN_6O_3S$) $[M + H]^+$, 520.17, found 521.2.

*5.1.23.(R)-2-((2-(3-aminopiperidin-1-yl)-7-(2-(methylsulfonyl)phenyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile (**12e**).*

Compound **12e** was prepared in a manner identical to that described for **12a** as light-yellow solid. Yield (two steps): 70.5%. ^{1}H NMR (400 MHz, MeOD) δ: 8.23-8.21 (d, J = 8.0 Hz, 1H), 7.86-7.82 (m, 1H), 7.78-7.74 (m, 1H), 7.70-7.68 (m, 2H), 7.65-7.61 (m, 1H), 7.26-7.22 (m, 1H), 7.00-6.97 (d, J = 9.2 Hz, 1H), 5.67-5.56 (m, 2H), 3.31 (s,

2H), 3.31 (s, 1H), 3.10-3.08 (m, 2H), 2.89 (s, 3H), 2.86-2.77 (m, 2H), 2.00-1.97 (m, 1H), 1.79-1.62 (m, 2H), 1.40-1.32 (m, 1H); ^{13}C NMR (125 MHz, MeOD) δ: 166.33, 164.30, 155.68, 154.39, 145.33, 145.26, 141.63, 139.81, 135.75, 134.59, 132.80, 129.71, 128.18, 127.48, 116.28, 115.26, 115.08, 114.92, 114.73, 113.56, 106.62, 55.38, 51.28, 46.95, 45.47, 41.89, 30.34, 22.26; ESI-MS calculated for ($C_{26}H_{25}FN_6O_3S$) $[M + H]^+$, 520.17, found 521.2.

*5.1.24.(R)-2-((2-(3-aminopiperidin-1-yl)-7-(3-(methylsulfonyl)phenyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile (**12f**).*

Compound **12f** was prepared in a manner identical to that described for **12a** as light-yellow solid. Yield (two steps): 72.8%. ^{1}H NMR (400 MHz, MeOD) δ: 8.98 (s, 1H), 8.31-8.29 (d, *J* = 7.6 Hz, 1H), 7.91 (s, 1H), 7.86-7.82 (m, 1H), 7.80-7.78 (d, *J* = 8.0 Hz, 1H), 7.66-7.62 (m, 1H), 7.24-7.19 (m, 1H), 6.91-6.89 (dd, *J* = 2.0 Hz, *J* = 8.4 Hz, 1H), 5.65-5.60 (m, 2H), 3.50-3.48 (m, 1H), 3.20 (s, 3H), 3.18 (s, 1H), 3.15-3.11 (m, 1H), 2.95-2.89 (m, 1H), 2.87-2.81 (m, 1H), 2.06-2.02 (m, 1H), 1.82-1.72 (m, 2H), 1.38-1.36 (m, 1H); ^{13}C NMR (125 MHz, MeOD) δ: 166.34, 164.31, 155.72, 154.33, 145.26, 140.89, 135.65, 135.57, 130.18, 129.22, 126.17, 124.52, 123.71, 116.26, 115.23, 115.05, 114.65, 114.38, 106.78, 57.28, 51.43, 46.98, 45.38, 43.15, 31.70, 22.99; ESI-MS calculated for ($C_{26}H_{25}FN_6O_3S$) $[M + H]^+$, 520.17, found 521.2.

*5.1.25.(R)-2-((2-(3-aminopiperidin-1-yl)-7-(4-(isopropylsulfonyl)phenyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile hydrochloride (**12g**).*

Compound **12g** was prepared in a manner identical to that described for **12a** as light-yellow solid. Yield (two steps): 58.5%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.48-12.47 (d, *J* = 2.8 Hz, 1H), 8.43-8.41 (d, *J* = 8.4 Hz, 2H), 8.32 (s, 3H), 8.13-8.12 (d, *J* = 3.2 Hz, 1H), 7.98-7.94 (m, 1H), 7.83-7.81 (d, *J* = 8.4 Hz, 2H), 7.36-7.31 (m, 1H), 7.04-7.01 (m, 1H), 5.55-5.39 (m, 2H), 3.54-3.51 (m, 1H), 3.44-3.42 (m, 1H), 3.40-3.37 (m, 1H), 3.15-3.07 (m, 2H), 2.87 (s, 1H), 1.98 (s, 1H), 1.84 (s, 1H), 1.62 (s, 2H), 1.19-1.17 (d, *J* = 26.8 Hz, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 166.87, 164.54, 154.88, 154.47, 147.85, 140.35, 140.03, 136.41, 133.35, 129.35, 127.79, 126.13, 117.13, 115.97, 115.49, 114.20, 107.10, 54.69, 51.87, 49.14, 46.67, 45.98, 27.25, 22.36, 15.74; ESI-MS calculated for ($C_{28}H_{29}FN_6O_3S$) $[M + H]^+$, 548.20, found 549.1.

*5.1.26.(R)-4-(2-(3-aminopiperidin-1-yl)-3-(2-cyano-5-fluorobenzyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-7-yl)benzenesulfonamide hydrochloride (**12h**).*

Compound **12h** was prepared in a manner identical to that described for **12a** as light-yellow solid. Yield (two steps): 60.8%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.40 (s, 1H), 8.29 (s, 3H), 8.29-8.27 (d, *J* = 8.4 Hz, 2H),

8.05-8.04 (d, *J* = 3.2 Hz, 1H), 7.98-7.95 (m, 1H), 7.83-7.81 (d, *J* = 4.2 Hz, 2H), 7.37-7.36 (m, 1H), 7.34-7.32 (m, 2H), 7.04-7.01 (m, 1H), 5.55-5.38 (m, 2H), 3.51-3.49 (m, 1H), 3.38-3.36 (m, 1H), 3.14-3.04 (m, 2H), 2.83 (s, 1H), 2.01-1.98 (m, 1H), 1.84 (s, 1H), 1.60-1.59 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 165.97, 163.95, 154.89, 154.31, 146.01, 141.15, 140.14, 137.92, 136.40, 127.24, 126.33, 126.01, 117.14, 115.95, 115.43, 114.70, 107.09, 52.80, 51.97, 46.76, 45.89, 28.05, 22.45; ESI-MS calculated for ($C_{25}H_{24}FN_7O_3S$) $[M + H]^+$, 521.16, found 522.1.

*5.1.27.(R)-2-((2-(3-aminopiperidin-1-yl)-7-(3-fluoro-4-(methylsulfonyl)phenyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile hydrochloride (**12i**).*

Compound **12i** was prepared in a manner identical to that described for **12a** as light-yellow solid. Yield (two steps): 60.8%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.57-12.56 (d, *J* = 2.4 Hz, 1H), 8.26 (s, 3H), 8.23-8.23 (m, 2H), 8.20-8.19 (d, *J* = 3.2 Hz, 1H), 7.98-7.94 (m, 1H), 7.85-7.81 (m, 1H), 7.37-7.32 (m, 1H), 7.07-7.04 (m, 1H), 5.55-5.38 (m, 2H), 3.51 (s, 1H), 3.39 (s, 1H), 3.33 (s, 3H), 3.07 (s, 2H), 2.86 (s, 1H), 2.00-1.98 (m, 1H), 1.84 (s, 1H), 1.60-1.59 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 163.94, 160.51, 158.52, 154.81, 143.07, 140.40, 136.41, 136.33, 129.74, 128.35, 124.68, 121.86, 117.11, 117.07, 115.97, 115.79, 115.37, 113.31, 107.05, 52.74, 51.95, 46.75, 46.07, 44.32, 27.77, 27.24; ESI-MS calculated for ($C_{26}H_{24}F_2N_6O_3S$) $[M + H]^+$, 538.16, found 539.1.

*5.1.28.(R)-2-((2-(3-aminopiperidin-1-yl)-7-(3-fluoro-4-(methylsulfonyl)phenyl)-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl)-4-fluorobenzonitrile (**12j**).*

Compound **12j** was prepared in a manner identical to that described for **12a** as light-yellow solid. Yield (two steps): 63.3%. ^{1}H NMR (400 MHz, MeOD) δ: 8.96 (s, 1H), 7.92 (s, 1H), 7.84 (m, 2H), 7.77 (s, 1H), 7.24 (s, 2H), 7.04-7.02 (d, *J* = 9.2 Hz, 1H), 5.68-5.56 (m, 2H), 3.61-3.58 (m, 1H), 3.45 (s, 1H), 3.20 (s, 3H), 3.20-3.13 (m, 2H), 2.94 (s, 1H), 2.16-2.14 (m, 1H), 1.89-1.83 (m, 2H), 1.62-1.60 (m, 1H); ^{13}C NMR (125 MHz, MeOD) δ: 166.30, 164.27, 157.75, 155.40, 154.25, 145.02, 141.18, 138.53, 135.74, 130.08, 129.60, 127.49, 122.84, 116.39, 115.38, 115.19, 114.40, 108.59, 106.78, 53.74, 51.71, 45.64, 43.01, 35.18, 28.80, 22.20; ESI-MS calculated for ($C_{26}H_{24}F_2N_6O_3S$) $[M + H]^+$, 538.16, found 539.1.

5.2. In vitro inhibition of DPP-4, DPP-8 and DPP-9

Solutions of test compounds at varying concentrations（final concentration ≤10 mM) were prepared in dimethyl sulfoxide (DMSO) and diluted into assay buffer containing 20 mM Tris (pH 7.4), 20 mM KCl, and 0.1 mg/mL BSA. Human DPP-IV (0.1 nM final concentration) was added to the dilutions and pre-incubated for 10 minutes at ambient temperature before the reaction was initiated by the addition of Gly-Pro-AMC

(H-glycyl-prolyl-7-amino-4-methylcoumarin, Sigma-Aldrich, 10 μM final concentration). The total volume of the reaction mixture was 100 μL. The kinetics of the reaction were monitored (excitation at 400 nm, emission at 505 nm) for 5-10 minutes, or an endpoint was measured after 10 minutes. Inhibition constants (IC_{50}) were calculated from enzyme progress curves using standard mathematical models.

5.3. In vitro DPP-4 inhibitory kinetic assay

Solutions of test compounds at varying concentrations（final concentration ≤10 mM) were prepared in dimethyl sulfoxide (DMSO) and diluted into assay buffer containing 20 mM Tris (pH 7.4), 20 mM KCl, and 0.1 mg/mL BSA. Human DPP-IV (0.1 nM final concentration) was added to the dilutions and pre-incubated for 10 minutes at ambient temperature before the reaction was initiated by the addition of Gly-Pro-AMC (H-glycyl-prolyl-7-amino-4-methylcoumarin, Sigma-Aldrich, 10 μM final concentration). The total volume of the reaction mixture was 100 μL. The kinetics of the reaction were monitored (excitation at 400 nm, emission at 505 nm) for 5-10 minutes, or an endpoint was measured after 10 minutes. Inhibition constants (IC_{50}) were calculated from enzyme progress curves using standard mathematical models.

5.4 In vitro hepatic stability study

Adult male SD rats (n= 4/group) were administered the test compounds dissolved in distilled water at a single dose of 25 mg/kg by oral administration and 5 mg/mL by injection. Blood samples of 100-200 μL were collected from the orbit at 11 time points within 24 hours. The blood concentration of the test compounds was determined by LC-MS/MS. The PK parameters were obtained from the pharmacokinetic software DAS. 2.0.

5.5 In vivo pharmacokinetic study

Adult male SD rats (n= 4/group) were administered the test compounds dissolved in distilled water at a single dose of 25 mg/kg by oral administration and 5 mg/mL by injection. Blood samples of 100-200 μL were collected from the orbit at 11 time points within 24 hours. The blood concentration of the test compounds was determined by LC-MS/MS. The PK parameters were obtained from the pharmacokinetic software DAS. 2.0.

5.6 In vivo efficacy study

Adult male ICR mice (at least n=4 per group) were orally gavaged with the test compounds dissolved in distilled water at a single dose of 1 mg/kg or 3 mg/kg. Blood samples of 20-25 μL were collected from the orbit at the time points indicated in Figure 6 for 24 hours and the plasma fraction was kept frozen until DPP-IV activity measurement. The plasma DPP-IV activity was determined by cleavage rate of Gly-Pro-AMC (H-glycyl-prolyl-7-amino-4-methylcoumarin; Sigma-Aldrich). Plasma (10 μL) was mixed

with 140 μL of 150 μM Gly-Pro-AMC in assay buffer that was composed of 25mM tris(hydroxymethyl)-aminomethane HCl (PH 7.4), 140 mM NaCl, 10 mM KCl and 0.1% bovine serum albumin, the fluorescence was determined by using Thermo Scientific Fluoroskan Ascent FL (excitation at 400 nm and emission at 505 nm). One unit of activity is defined as the amount of enzyme that produces 1 μM products per minute. The DPP-IV relative activity in plasma was described as activity (indicated time points)/activity (initial point).

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